DEVELOPMENT OF THE AMPHIBIAN EAR RUDIMENT IN EXPLANTS

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In connection with studies on the development of the cartilage capsule surrounding the auditory vesicle (Kaan, 1930), the ear rudiment of *Ambystoma maculatum* was transplanted into various regions of the young larva. These transplanted rudiments developed into relatively normal labyrinths only in the region immediately anterior to the normal ear. In other parts of the body, the transplants formed simple vesicles with or without sensory epithelium. The endolymphatic duct was absent and there was no indication of semicircular canals. Somewhat greater development occurred in ear rudiments of *Rana sylvatica* when transplanted into *Ambystoma* larvae. In later experiments (Kaan, 1938), dealing with the origin of the cartilage capsule, abnormalities occurred in the developing ear *in situ*. When the size of the ear region was reduced following the insertion of other tissues, the resulting labyrinth was reduced in size. When the capsule was defective, the corresponding portion of the labyrinth expanded into the available space, tending to form a cystic vesicle.

The occurrence of these developmental anomalies brought up the question to what extent the ear rudiment possessed an intrinsic capacity for differentiation, and it was, therefore, of interest to determine whether and how far it would develop when removed from the influence of the surrounding tissues and of the organism as a whole. Preliminary experiments indicated that an ear vesicle would develop in explants cultured in a sterile salt solution and consequently, two series of experiments were undertaken in the spring of 1945 at Stanford University on larvae of *Taricha rivularis* and *T. torosa*. Subsequent experiments were conducted in 1960 1961 and 1962 at the Marine Biological Laboratory on *Ambystoma maculatum* and, to a limited extent, on *A. tigrinum* and *Rana sylvatica*. Results of these experiments indicate that in all of the species the ear rudiment can develop in an explant enclosed wholly or partially by epithelium and accompanied by more or less of the mesendoderm and mesectoderm which normally surrounds it. Such an auditory vesicle can form an endolymphatic duct and areas of sensory epithelium and it is commonly associated with a ganglion, the eighth cranial (Yntema, 1937). The most advanced degree of differentiation attained by these explanted ear rudiments appears similar to that of normal salamander larvae at Stage 40 (Harrison) (Fig. 1) and of frog larvae at Stage 22 (Pollister and Moore, 1937) (Fig. 2). These stages represent the period of development when the individual cells of the labyrinth have utilized all of the nourishment within their own yolk granules and are dependent on material brought by the circulatory system. Differentiation of the

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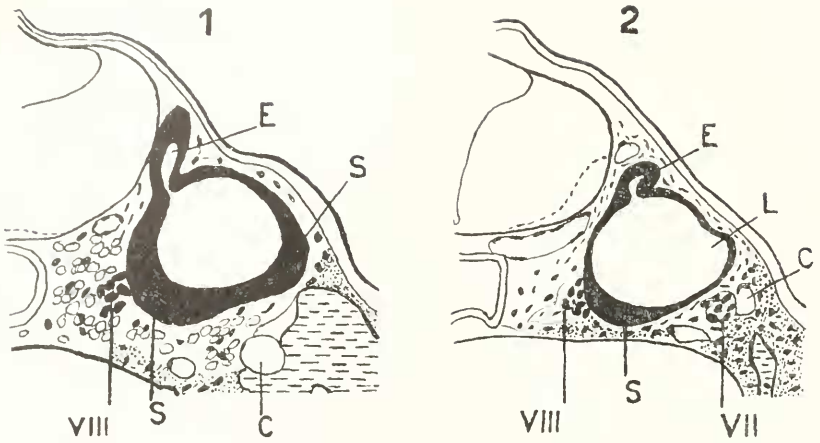


FIGURES 1 and 2. Normal left labyrinths of donor larvae.

FIGURE 1. EM-77, *Ambystoma maculatum*, Stage 40– (Harrison).

FIGURE 2. E$_s$-5, *Rana sylvatica*, Stage 22–23 (Pollister and Moore). These and the subsequent figures are tracings of camera lucida drawings at a magnification of 100×. They are represented semi-diagrammatically, with auditory vesicles in solid black, cartilage in fine stippling, muscle in short, horizontal lines; nuclei of cells are in solid black and yolk granules in open circles; inner and outer edges of surface epithelium are indicated by solid lines. E—Endolymphatic duct; S—Sensory epithelium; L—Pouch of lateral canal; C—Capillary; VII and VIII—Ganglia of seventh and eighth cranial nerves.

ear rudiment and of the surrounding tissues appear to be independent of each other except as developing endothelial sinuses may exert mechanical pressure within the confined space of the explant.

In recent years, the nomenclature of some species of salamanders has been changed and, since these are the animals most commonly used in embryological experiments, a certain amount of confusion of terminology exists in the literature. Throughout this paper, the author has used the nomenclature for each species as given by Gentry (1955) and Twitty (1959).

I wish at this time to express my sincere appreciation to Dr. Victor C. Twitty for his kindness in making the facilities of his laboratory available to me and for his suggestions concerning methods of technique.

MATERIAL AND METHODS

Two series of operations were performed on larvae of each of the five species. In the E Series, ectoderm with attached ear rudiment was cut from the right side of the head and an attempt was made to remove all of the mesendoderm from the inner surface of the excised piece, particularly those cells which were close to the ear rudiment. Varying amounts of neural crest were also removed. In the EM Series, both the mesendoderm and neural crest were left intact. Some variation occurred in the size of the piece of ectoderm but, in general, cuts were made in

the hyomandibular groove and along the dorsal mid-line, with parallel cuts in the presomite and gill regions to include a piece of ectoderm approximately 0.6 mm. square. This provided enough ectoderm to completely enclose the ear rudiment and any surrounding tissues. Preliminary experiments had shown that degeneration occurred rapidly in explants with a small amount of ectoderm, leaving the ear rudiment exposed to the salt solution. Operations on the salamander larvae included Stages 23 through 32, with the majority at Stages 25 through 29. These represented development of the ear rudiment from the time just prior to formation of the placode through closure of the cup to form the otic vesicle. Frog larvae were operated on at Stages 16 and 17, corresponding to Stages 25 and 27 in the salamanders. Each experiment was continued as long as the explant appeared to be in a healthy condition. Table I summarizes the experiments in each series.

In 1962, 35 operations were performed on larvae of *A. maculatum* in which a portion of the lateral wall of the myelencephalon was included in the explant. Although several methods were used, the results were unsuccessful. The cut edges of the myelencephalon rolled outward, exposing the inner lining or the developing nerve cells and the ectoderm would not grow over this brain tissue. In most cases, the explant disintegrated rapidly; in others, either the piece of brain wall was healed out or the entire contents healed out, leaving a hollow ball of cells.

The sterile technique developed by Twitty was used in performing the operations and in the subsequent culture of the explants in modified Holtfreter's solution (Twitty, 1945). The operating instruments were finely-sharpened iridectomy scissors and steel needles. Both the donor larvae and the explant were transferred from the wax operating dishes, after 15–30 minutes, to an autoclaved glass stender dish and fresh salt solution. They were then left together in the same dish so that the experimental and control auditory vesicles would be subjected to the same

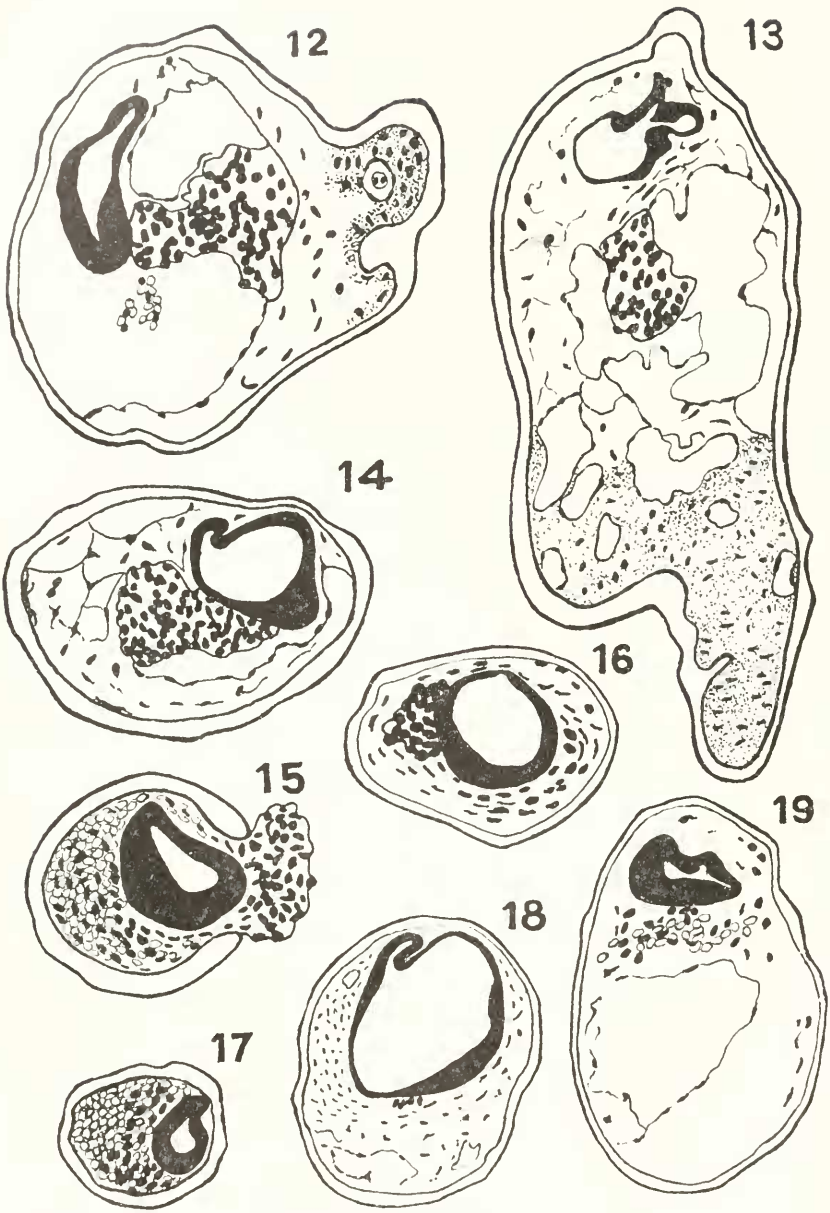
TABLE I

Summary of the E and EM series of explants in each of the five species

The individual species are distinguished as follows: E_r and EM_r—*Taricha rivularis*; E_t and EM_t—*T. torosa*; E and EM—*Ambystoma maculatum*; E_a and EM_a—*A. tigrinum*; E_s and EM_s—*Rana sylvatica*

Series	Total no. of operations	Total no. of surviving explants	Number of explants studied	Stages at operation	Stages at preservation	Length of culture periods (no. of days)
E_r	36	28	25	24–29	37–45	15–26
E_t	58	40	31	24–32	41–45	12–24
E	37	12	10	24–29	39–42	6–12
E_a	10	8	7	25–29	33–38	3–6
E_s	6	3	3	17(27)*	22(40)*	4–6
EM_r	49	30	23	23–31	37–42	12–26
EM_t	48	37	28	24–31	40–45	14–26
EM	33	22	19	23–29	37–44	6–20
EM_a	10	7	6	25–29	34–36	2–8
EM_s	12	9	9	16–17 (25–27)*	20–23 (38–40+)*	3–6

* Figures in parentheses indicate the approximately comparable stage in salamander larvae.



FIGURES 12 through 19. Explants from the EM Series.

FIGURE 12. EM_t-22, *T. torosa;* operated at Stage 28; preserved after 14 days; larva at Stage 40. Vesicle is flattened by pressure of fluid in the large endothelial sinus but degree of differentiation is comparable to the normal left vesicle. Capillary within the rudimentary gill (right) contains a myelocyte.

FIGURE 13. EM_t-44, *T. vivularis;* operated at Stage 27; preserved after 26 days; larva not kept. Vesicle is compressed and distorted. Solid projection from upper part of vesicle is

endolymphatic ducts and sensory areas could be identified (Figs. 5, 6, 7, 12, 14). Auditory vesicles of *T. rivularis* showed relatively little differentiation in explants of the E_r Series (Figs. 3, 4); it was difficult to determine either endolymphatic ducts or sensory areas. In vesicles of the EM_r Series, sensory areas were more distinct but endolymphatic ducts were very rudimentary or apparently absent (Fig. 13).

A number of exploratory operations, including both the E and EM types of explants, were performed on *A. maculatum* larvae prior to 1945. Since these operations, as well as the culture methods, were not standardized, only those experiments performed in 1960–1962 will be considered in this report (E-44 through E-81 and EM-47 through EM-80). Of these experiments, E-44 through E-63 and EM-47 through EM-76 were from eggs collected in the vicinity of Falmouth, Massachusetts; the remainder were from eggs shipped from Tennessee. There was no obvious difference in the development of larvae and explants from these two locations. There was also no clear indication that variations in the temperature at which the explants were cultured had any specific effect on the differentiation of the vesicles. Although the mortality was higher in the E Series, auditory vesicles in the surviving explants showed somewhat greater differentiation than those in the EM Series (Figs. 8, 9, 16). Figures 17 and 19 represent sections of two explants which developed from operations at stage 23, just prior to the appearance of the auditory placode. The vesicles are smaller than those from stages in which the ear rudiment is distinct but they show the beginnings of differentiation. Since another vesicle regenerated at the site from which the explant, EM-77, was removed, there is a question as to how much of the potential ear tissue was represented in the explanted vesicle. An interesting point which needs further investigation is that, in contrast to the explanted ear, the regenerated ear was very close in size and degree of differentiation to the unoperated right ear (Fig. 1).

Explants of *A. tigrinum* cannot be strictly compared with those of the other salamanders because their number was very limited and they were, of necessity, preserved at much younger stages (Stages 33–36). In both the E_a and EM_a groups, however, the degree of development of the explanted vesicles was directly

attached to surface epithelium in other sections. Endolymphatic duct cannot be definitely identified. Ganglion has only a slender attachment to vesicle. Section is through one of several "gills."

FIGURE 14. EM_t-8, *T. torosa;* operated at Stage 27; preserved after 18 days; larva at Stage 43. This is typical of explants in which there was no large accumulation of fluid. Pouch of lateral canal may have started.

FIGURE 15. EM_a-7, *A. tigrinum;* operated at Stage 27; preserved after 5 days; larva at Stage 35+. Vesicle is comparable to the left vesicle and shows the beginning of the endolymphatic duct and of sensory epithelium. The ganglion is not covered with ectoderm.

FIGURE 16. EM-55, *A. maculatum;* operated at Stage 29; preserved after 11 days; larva at Stage 40. This is typical of explants in this group. A rudimentary endolymphatic duct is present in other sections. Arrangement of cells around the vesicle suggests the beginning of capsule formation.

FIGURE 17. EM-77, *A. maculatum;* operated at Stage 23; preserved after 8 days; larva at Stage 40–. Normal left labyrinth shown in Figure 1. A second labyrinth regenerated on the right side at the site of the operation.

FIGURE 18. EM_s-7, *R. sylvatica;* operated at Stage 17+; preserved after 6 days; larva at Stage 23+ (Stage 3 of Birkman, 1940). Pouch of lateral canal is present. Vesicle is comparable to normal vesicle of Figure 2.

FIGURE 19. EM-80, *A. maculatum;* operated at Stage 23; preserved after 13 days; larva at Stage 41. Vesicle is compressed but shows greater differention than in EM-77.

comparable to that of the corresponding unoperated ears. The beginning sensory areas were indicated (Fig. 10) as well as the initial elongation which precedes the formation of the endolymphatic duct (Fig. 15).

Rana sylvatica larvae presented special conditions because their development was much faster than that of the salamanders. In less than one week, the auditory vesicles reached a degree of differentiation which required from two to three weeks in salamander larvae (Figs. 11, 18). It is interesting to note, however, that none of the explanted vesicles reached the same stage as the corresponding left vesicles which remained in the larvae. A comparison of Figure 11, showing the explanted right vesicle of E_s-5, with Figure 2, which represents the unoperated left vesicle of the same larva, brings out this difference.

Certain facts appear evident from a study of all of the explants: the explanted ear rudiment, when cultured in a balanced sterile salt solution, can differentiate to the extent of forming an endolymphatic duct and recognizable sensory areas; it can give rise to the eighth cranial ganglion; it does not develop beyond the equivalent of the salamander stage 40 or *Rana* stage 23, regardless of the length of the culture period and the degree of development of the ear in the donor larva.

The appearance and differentiation of tissues surrounding the explanted vesicles were of considerable interest, although they were not the primary concern of these experiments. The epithelium covering the explants varied from very thick, sometimes in solid masses, to a very thin, single layer over the distended areas. Typically, it consisted of the two layers of cells which are characteristic of the Amphibia and showed the same degree of development as the epithelium in the donor larvae. As the explant got older, an increasing number of cells in the outer epithelial layer became enlarged and vacuolated, presumably from the accumulation of fluid, and one of the signs of approaching disintegration of the explant was the rough appearance of the epithelium produced by these bulging cells. In the *Rana* explants suckers developed in the epithelium and produced mucus at about the same time as the suckers in the donor larvae.

Neural crest cells differentiated into sense organs of the lateral line system which could be distinguished in the surface epithelium (Stone, 1922). Pigment cells lying just beneath the epithelium undoubtedly arose from the neural crest as did the mesenchyme in some explants of the E Series (Figs. 6, 7, 8). Some of the mesenchyme may have come from mesendoderm cells which were not removed at the time of the operation. Presumably, mesendoderm gave rise, also, to the capillaries and endothelial sinuses of both series, as well as the muscle and blood cells which were present in certain explants of the EM Series. Origin of the cartilage which developed in some explants of the EM_r and EM_t groups (Figs. 12, 13) was not so obvious. However, it appeared only in the gill-like projections and this would indicate that it represented branchial arch material arising from mesectoderm (Stone, 1922). A careful study of all explants showed one in the EM group (Fig. 16), two in the EM_t group and two or three in the EM_s group (Fig. 18) in which the arrangement of cells next to the auditory vesicle suggested the formation of precartilage. This is particularly interesting in view of the fact that mesendoderm, the source of the auditory capsule (Stone, 1922, 1929; Mangold, 1937), was included in all explants of the EM Series.

It was apparent, then, that differentiation of the ear rudiments within the

explants occurred independently of the surrounding tissues. These tissues did, however, influence the development of the ears to the extent that an epithelial covering prevented degeneration of the rudiment and that pressure from distended epithelial sinuses flattened or otherwise distorted the shape of the developing vesicle (Figs. 5, 9, 12, 13, 19). In the most extreme cases, ear rudiments were flattened against the surface epithelium and were unable to form vesicles.

Discussion

Stages 39 through 41 in the salamander and Stages 22–23 in the frog marked the apparent limit of development of the explanted ear rudiments. This period, just preceding the formation of the definitive labyrinth, is the time when yolk granules disappear from the individual cells of the auditory vesicle. The energy for further development, therefore, must come from sources outside the ear itself, since its intrinsic food supply has presumably been completely utilized. The fact that this period is a critical one has been brought out by investigations of several aspects of larval development.

Copenhaver (1926) observed completion of circulation in the gills of *A. maculatum* and an increase in rate of the heartbeat at Stages 36–37. A further increase in heart rate occurred at Stages 40–41. Pollister and Moore (1937) noted circulation in the gills of *R. sylvatica* at a comparable stage (Stage 20). In a comparison of *A. maculatum* and *A. tigrinum*, Hopkins and Handford (1943) recorded a gradual rise in oxygen consumption in both species up to Stage 37, with a more rapid rate of increase at the beginning of heart beat and muscular movement. The rate in *A. tigrinum* was higher than that in *A. maculatum*. Connon (1947) showed that, at a temperature of 20°, the respiratory rate of *T. torosa* was consistently higher than that of *T. rivularis* and exhibited a greater and more rapid increase. The curves in both species increased gradually during embryonic and early larval stages and more rapidly during later stages. He believed that there was a positive correlation between the developmental and respiratory rates, and noted that the yolk was used up more rapidly in *T. torosa*. Løvtrup (1953), using eggs, embryos and larvae of the axolotl (*A. mexicanum*), made a series of determinations of the utilization of carbohydrate, fat and protein at the different stages of development. He found that rate of carbohydrate consumption reached its peak during the neurula stages and then began to decline. Fat consumption began during the neurula stages and its rate increased during the larval stages. Thus, beginning with Stage 36, the rate of carbohydrate consumption was decreasing and the rate of fat consumption was increasing. Yolk, included within platelets or granules in the individual cells, contained a phospholipoprotein but no reducing carbohydrate. Using phosphoprotein phosphorus as an indication of the presence of yolk, he recorded a continual decrease, beginning soon after Stage 20. In a later, more detailed study (Løvtrup, 1955), he measured the activity of a series of enzymes, as well as RNA and DNA, in the embryos and larvae of *A. mexicanum*, *A. maculatum*, *R. platyrrhinus* and *Xenopus laevis*. He found that, for several of the substances tested, there was a steep rise in activity at about Stage 40 in *A. mexicanum* and *A. maculatum* and at Stages 21–23 in *R. platyrrhinus*. He concluded that this rise, coupled with the decrease in phosphoprotein phosphorus, indicated the end of the yolk reserve and

the beginning of synthesis of new substances. This increased activity was correlated with the differentiation of liver and intestine, and the establishment of the vitelline circulation. Somewhat in the nature of a corollary to Løvtrup's experiments were those of Eyal-Giladi and Eyal (1962). They placed eggs and larvae of the axolotl in varying concentrations of chlorpromazine for varying lengths of time and determined the effects on development after removal to a solution of sulfadiazine and streptomycin. Among a variety of actions, chlorpromazine causes retardation of growth without malformations and the authors found that, under certain conditions, chlorpromazine permitted development up to Stages 37–38, followed by cessation of development and degeneration. They considered that these were critical stages with respect to oxygen consumption and, thus, particularly susceptible to the action of chlorpromazine.

Results of the foregoing experiments may be applied directly to the results obtained with the explanted ear rudiments. The most advanced stages of development reached by these auditory vesicles represent the limits of differentiation which can be attained through utilization of the yolk material in the individual cells. In normal larvae at comparable stages, there is an increase in respiratory rate and in oxygen consumption; changes in the nature of the food supply following depletion of the yolk involve the synthesis of new substances by enzymes in the developing digestive tract. The auditory vesicle is, therefore, dependent upon sources outside itself for the increased oxygen and food requirements necessary for further development and differentiation. At this time, the accelerated rate of the heartbeat and the establishment of the early circulatory system afford the means by which this is accomplished in the larva. Replacement of the salt solution by an adequate nutrient solution would be necessary to provide suitable conditions for further ear development in explants.

Detwiler and Van Dyke (1950) concluded that the results of transplantation experiments supported their view that ear rudiments normally depended upon the presence of the medulla for their differentiation. Confirmation of these results was not possible in the present series of experiments, since no explants were obtained in which a portion of the medulla was included. However, Mangold (1937) did succeed in getting viable explants from *Triton alpestris*, *T. taeniatus* and *A. mexicanum* which contained some brain tissue. These were all taken from neurula stages, prior to closure of the neural folds and formation of a definite ear rudiment. Otic vesicles appeared in some of the explants and, although none of them was complete, some did show endolymphatic duct, sensory areas, ganglion and the beginnings of definitive canals. Mangold believed that the presence of the medullary folds was a necessary and determining factor in the first appearance of the ear rudiment, but he was surprised that complete labyrinths did not develop, since the spatial conditions in the explant were favorable and the normal adjacent tissues were present. The present series of explants, containing a distinct ear rudiment, show that an auditory vesicle with endolymphatic duct, sensory areas and ganglion can be produced in the absence of medullary tissue and with minimum of other surrounding tissues, if the developing ear is enclosed by ectodermal epithelium. Further investigation is needed to determine definitely whether the formation of a complete auditory labyrinth is dependent upon the presence of the myelencephalon, the development of a cartilage

capsule from the surrounding mesendoderm, or whether the ear is capable of still further independent development and differentiation in the presence of specific and essential nutrients.

In conclusion, then, it can be stated that explants of the ear rudiments of *T. rivularis*, *T. torosa*, *A. maculatum*, *A. tigrinum* and *R. sylvatica* are capable of undergoing development and differentiation when cultured in a sterile balanced salt solution. It appears to be essential that they be covered by ectoderm but their development is fundamentally independent of other surrounding tissues except as these exert mechanical pressure on the vesicle. Variations in temperature have no obvious effect on either mortality of the explant or development of the ear. Under these conditions, the ear rudiment may form a vesicle with endolymphatic duct and sensory epithelium and, in some cases, the beginning of the lateral pouch and separation of the sensory epithelium into two or more areas. The degree of differentiation of such a vesicle corresponds to about Stage 40 in salamander larvae and Stage 22 in the frog, a period in larval development when the individual cells of the ear have used up all of their intrinsic food material and are dependent on extrinsic sources for the nutrients needed in further development and differentiation. This does not necessarily represent the full capacity of the ear for independent differentiation. The exact nutritional requirements for expressing its ultimate potentiality may be determined through further experimentation with nutrient culture media.

Summary

1. Two series of operations were performed on larvae of *Taricha rivularis*, *T. torosa*, *Ambystoma maculatum*, *A. tigrinum* and *Rana sylvatica*. In the E Series, a piece of ectoderm with attached ear rudiment was excised, and the mesendoderm and some, if not all, of the mesectoderm were removed. In the EM Series, mesendoderm and mesectoderm were included with the ear rudiment and ectoderm. These excised tissues were cultured, together with the donor larvae, in sterile Holtfreter's solution for varying lengths of time and, in most cases, at 15° or 16° C.

2. Study of serial sections of the explants showed that the ear rudiments of *T. rivularis*, *T. torosa*, *A. maculatum* and *R. sylvatica* were capable of independent development and differentiation up to the stage when the auditory vesicle possesses a distinct endolymphatic duct and sensory epithelium. This corresponds to Stage 40 in the salamanders and Stage 22 in the frog. In a few cases, there was some indication of the beginning of formation of a lateral pouch and separation of two areas of sensory epithelium. The explants of *A. tigrinum* were cultured for only a short period and consequently showed only the earliest signs of differentiation.

3. At Stages 40 (salamander) and 22 (frog), the cells of the auditory vesicles no longer contain visible yolk granules. They have, presumably, used up all of their intrinsic food material and are dependent on the developing circulatory and digestive systems for the supply of further nutrients.

4. A review of the literature indicates that these stages in the developing amphibian larva are marked by an increase in the heart rate and the rate of oxygen consumption, as well as an increase in the activity of specific enzymes which

synthesize new food materials to replace those originally present in the yolk granules.

5. It is concluded that the degree of differentiation attained by the ear rudiment when cultured as an explant in a balanced salt solution may not necessarily represent the limit of its potentiality for independent development. Further development might be possible if the explant were cultured in an adequate nutrient solution.

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